Based on our results, we believe that the production of gastric lesions in HSF1-null mice is due to their inability to express protective HSPs, leading to apoptosis of the gastric mucosal cells. Although oral administration of ethanol led to the production of gastric lesions, there was a concomitant up-regulation of HSPs (Figs. 2 and 3), with significantly fewer apoptotic cells being recorded in wild-type mice than in HSF1-null mice (Fig. 4). Induction of necrosis by ethanol may also be stimulated in HSF1-null mice, because up-regulation of HSPs made gastric mucosal cells resistant to ethanol-induced necrosis (Tomisato et al., 2001). Other factors that are believed to be involved in the production of gastric lesions, including gastric acid secretion, GMBF and PGE_2 levels, were similar in both wild-type and HSF1-null mice (Fig. 6). Artificial pre-induction of HSPs renders cultured gastric mucosal cells resistant to ethanol-induced apoptosis (Mizushima et al., 1999). Among the various HSPs tested, oral administration of ethanol up-regulated only HSP70 in terms of protein level (Fig. 2B). Furthermore, HSP70 is thought to be the major anti-apoptotic HSP; either HSP70 binds to Apaf-1, thereby preventing activation of caspases, or HSP70 suppresses the apoptotic pathway downstream of caspase-3 activation and apoptosis-inducing factor (AIF)-induced

chromatin condensation (Beere et al., 2000; Jaattela et al., 1998; Ravagnan et al., 2001; Saleh et al., 2000). However, despite the apparent significance of HSP70, it should still be noted that loss of the *hsf1* gene also decreased the background level of other HSPs (Fig. 2B), which may play some role in the HSF1-dependent protection of the gastric mucosa. It is also possible that the production of gastric lesions in HSF1-null mice involves other mechanisms, suggested in recent papers (Fujimoto et al., 2005; Inouye et al., 2003). For example, HSF1-null mice display elevated levels of tumor necrosis factor (TNF)- , a pro-inflammatory cytokine, and are susceptible to increased mortality following endotoxic or inflammatory challenge (Wirth et al., 2004; Xiao et al., 1999). Given that it is well known that pro-inflammatory cytokines, including TNF- stimulate the production of gastric lesions, it remains possible that the development of such lesions in HSF1-null mice involves elevated levels of TNF- . In addition, involvement of iNOS is also possible, because it has been shown that iNOS is involved in tissue damage and over-expression of HSP70 has been shown to inhibit iNOS and ameliorate the damage (Kiang, 2004; Pittet et al., 2002).

GGA has attracted considerable attention as an HSP-inducer, largely due to its clinical value as an anti-ulcer drug and because it can induce HSPs without affecting cell viability (Hirakawa et al., 1996). GGA has been suggested to play a protective role through HSP-induction in a variety of disease states; oral administration of GGA up-regulates HSPs in brain and heart and exerts a protective effect against spinal and bulbar muscular atrophy, cerebral ischemia and ischemic heart disease (Katsuno et al., 2005; Ooie et al., 2001; Yasuda et al., 2005). However, no previous reports have shown that the HSP-inducing activity of GGA contributes to these clinically beneficial outcomes, including its anti-ulcer effects. In this study, using immunohistochemical analysis, we have demonstrated that oral administration of GGA alone up-regulates gastric mucosal HSP70, and that pre-administration of GGA stimulates the ethanol-induced up-regulation of HSP70. Furthermore, we have revealed that pre-administration of GGA suppresses gastric lesions in wild-type mice but not in HSF1-null mice. These results argue strongly in favour of the HSP-inducing activity of GGA contributing to its anti-ulcer effects, providing the first direct genetic link between the pharmacological behaviour of the drug and the resultant clinical outcome.

In summary, this study provides direct genetic evidence suggesting that HSPs, following their HSF1-dependent up-regulation, confer protection against the development of gastric lesions.

References

Araki H, Ukawa H, Sugawa Y, Yagi K, Suzuki K and Takeuchi K (2000) The roles of prostaglandin E receptor subtypes in the cytoprotective action of prostaglandin E2 in rat stomach. *Aliment Pharmacol Ther* **14 Suppl 1**:116-124.

Beere HM, Wolf BB, Cain K, Mosser DD, Mahboubi A, Kuwana T, Tailor P, Morimoto RI, Cohen GM and Green DR (2000) Heat-shock protein 70 inhibits apoptosis by preventing recruitment of procaspase-9 to the Apaf-1 apoptosome. *Nat Cell Biol* **2**:469-475.

Christians E, Davis AA, Thomas SD and Benjamin IJ (2000) Maternal effect of Hsf1 on reproductive success. *Nature* **407**:693-694.

Ebert MP, Schafer C, Chen J, Hoffmann J, Gu P, Kubisch C, Carl-McGrath S, Treiber G, Malfertheiner P and Rocken C (2005) Protective role of heat shock protein 27 in gastric mucosal injury. *J Pathol* **207**:177-184.

Filaretova L, Tanaka A, Miyazawa T, Kato S and Takeuchi K (2002) Mechanisms by which endogenous glucocorticoid protects against indomethacin-induced gastric injury in rats. *Am J Physiol Gastrointest Liver Physiol* **283**:G1082-1089.

Fujimoto M, Takaki E, Hayashi T, Kitaura Y, Tanaka Y, Inouye S and Nakai A (2005) Active HSF1 significantly suppresses polyglutamine aggregate formation in cellular and mouse models. *J Biol Chem* **280**:34908-34916.

Furutani K, Aihara T, Nakamura E, Tanaka S, Ichikawa A, Ohtsu H and Okabe S (2003) Crucial role of histamine for regulation of gastric acid secretion ascertained by histidine decarboxylase-knockout mice. *J Pharmacol Exp Ther* **307**:331-338.

Futaki N, Arai I, Hamasaka Y, Takahashi S, Higuchi S and Otomo S (1993) Selective inhibition of NS-398 on prostanoid production in inflamed tissue in rat carrageenan-air-pouch inflammation. *J Pharm Pharmacol* **45**:753-755.

Gething MJ and Sambrook J (1992) Protein folding in the cell. *Nature* **355**:33-45.

Hirakawa T, Rokutan K, Nikawa T and Kishi K (1996) Geranylgeranylacetone induces heat shock proteins in cultured guinea pig gastric mucosal cells and rat gastric mucosa. *Gastroenterology* **111**:345-357.

Holzer P (1998) Neural emergency system in the stomach. *Gastroenterology* **114**:823-839.

Inouye S, Izu H, Takaki E, Suzuki H, Shirai M, Yokota Y, Ichikawa H, Fujimoto M and Nakai A (2004) Impaired IgG production in mice deficient for heat shock transcription factor 1. *J Biol Chem* **279**:38701-38709.

Inouye S, Katsuki K, Izu H, Fujimoto M, Sugahara K, Yamada S, Shinkai Y, Oka Y, Katoh Y and Nakai A (2003) Activation of heat shock genes is not necessary for protection by heat shock transcription factor 1 against cell death due to a single exposure to high temperatures. *Mol Cell Biol* **23**:5882-5895.

Itoh YH and Noguchi R (2000) Pre-treatment with mild whole-body heating prevents gastric ulcer induced by restraint and water-immersion stress in rats. *Int J Hyperthermia* **16**:183-191.

Jaattela M (1999) Escaping cell death: survival proteins in cancer. *Exp Cell Res* **248**:30-43.

Jaattela M, Wissing D, Kokholm K, Kallunki T and Egeblad M (1998) Hsp70 exerts its anti-apoptotic function downstream of caspase-3-like proteases. *EMBO J* **17**:6124-6134.

Katsuno M, Sang C, Adachi H, Minamiyama M, Waza M, Tanaka F, Doyu M and Sobue G (2005) Pharmacological induction of heat-shock proteins alleviates polyglutamine-mediated motor neuron disease. *Proc Natl Acad Sci U S A* **102**:16801-16806.

Kiang JG (2004) Inducible heat shock protein 70 kD and inducible nitric oxide synthase in hemorrhage/resuscitation-induced injury. *Cell Res* **14**:450-459.

Kiang JG and Tsokos GC (1998) Heat shock protein 70 kDa: molecular biology, biochemistry, and physiology. *Pharmacol Ther* **80**:183-201.

Kunisaki C and Sugiyama M (1992) Effect of teprenone on acute gastric mucosal lesions induced by cold-restraint stress. *Digestion* **53**:45-53.

Mathew A and Morimoto RI (1998) Role of the heat-shock response in the life and death of proteins. *Ann N Y Acad Sci* **851**:99-111.

McMillan DR, Xiao X, Shao L, Graves K and Benjamin IJ (1998) Targeted disruption of heat shock transcription factor 1 abolishes thermotolerance and protection against heat-inducible apoptosis. *J Biol Chem* **273**:7523-7528.

Miller TA (1983) Protective effects of prostaglandins against gastric mucosal damage: current knowledge and proposed mechanisms. *Am J Physiol* **245**:G601-623.

Mizushima T, Tsutsumi S, Rokutan K and Tsuchiya T (1999) Suppression of ethanol-induced apoptotic DNA fragmentation by geranylgeranylacetone in cultured guinea pig gastric mucosal cells. *Dig Dis Sci* **44**:510-514.

Morimoto RI (1998) Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. *Genes Dev* **12**:3788-3796.

Murakami M, Oketani K, Fujisaki H, Wakabayashi T and Ohgo T (1981) Antiulcer effect of geranylgeranylacetone, a new acyclic polyisoprenoid on experimentally induced gastric and duodenal ulcers in rats. *Arzneimittelforschung* **31**:799-804.

Nakamura K, Rokutan K, Marui N, Aoike A and Kawai K (1991) Induction of heat shock proteins and their implication in protection against ethanol-induced damage in cultured guinea pig gastric mucosal cells. *Gastroenterology* **101**:161-166.

Ooie T, Takahashi N, Saikawa T, Nawata T, Arikawa M, Yamanaka K, Hara M, Shimada T and Sakata T (2001) Single oral dose of geranylgeranylacetone induces heat-shock protein 72 and renders protection against ischemia/reperfusion injury in rat heart. *Circulation* **104**:1837-1843.

Otani S, Otaka M, Jin M, Okuyama A, Itoh S, Iwabuchi A, Sasahara H, Itoh H, Tashima Y and Masamune O (1997) Effect of preinduction of heat shock proteins on acetic acid-induced colitis in rats. *Dig Dis Sci* **42**:833-846.

Pittet JF, Lu LN, Geiser T, Lee H, Matthay MA and Welch WJ (2002) Stress preconditioning attenuates oxidative injury to the alveolar epithelium of the lung following haemorrhage in rats. *J Physiol* **538**:583-597.

Ravagnan L, Gurbuxani S, Susin SA, Maisse C, Daugas E, Zamzami N, Mak T, Jaattela M, Penninger JM, Garrido C and Kroemer G (2001) Heat-shock protein 70 antagonizes apoptosis-inducing factor. *Nat Cell Biol* **3**:839-843.

Saika M, Ueyama T and Senba E (2000) Expression of immediate early genes, HSP70, and COX-2 mRNAs in rat stomach following ethanol ingestion. *Dig Dis Sci* **45**:2455-2462.

Saleh A, Srinivasula SM, Balkir L, Robbins PD and Alnemri ES (2000) Negative regulation of the Apaf-1 apoptosome by Hsp70. *Nat Cell Biol* **2**:476-483.

Takano T, Tsutsumi S, Tomisato W, Hoshino T, Tsuchiya T and Mizushima T (2002) Geranylgeranylacetone protects guinea pig gastric mucosal cells from gastric stressor-induced apoptosis. *Dig Dis Sci* **47**:1546-1553.

Takeuchi K, Kato S, Takeeda M, Ogawa Y, Nakashima M and Matsumoto M (2003) Facilitation by endogenous prostaglandins of capsaicin-induced gastric protection in rodents through EP2 and IP receptors. *J Pharmacol Exp Ther* **304**:1055-1062.

Terano A, Hiraishi H, Ota S and Sugimoto T (1986) Geranylgeranylacetone, a novel anti-ulcer drug, stimulates mucus synthesis and secretion in rat gastric cultured cells. *Digestion* **33**:206-210.

Tomisato W, Takahashi N, Komoto C, Rokutan K, Tsuchiya T and Mizushima T (2000) Geranylgeranylacetone protects cultured guinea pig gastric mucosal cells from indomethacin. *Dig Dis Sci* **45**:1674-1679.

Tomisato W, Tsutsumi S, Hoshino T, Hwang HJ, Mio M, Tsuchiya T and Mizushima T (2004) Role of direct cytotoxic effects of NSAIDs in the induction of gastric lesions. *Biochem Pharmacol* **67**:575-585.

Tomisato W, Tsutsumi S, Tsuchiya T and Mizushima T (2001) Geranylgeranylacetone protects guinea pig gastric mucosal cells from gastric stressor-induced necrosis by induction of heat-shock proteins. *Biol Pharm Bull* **24**:887-891.

Tsutsumi S, Tomisato W, Takano T, Rokutan K, Tsuchiya T and Mizushima T (2002) Gastric irritant-induced apoptosis in guinea pig gastric mucosal cells in primary culture. *Biochim Biophys Acta* **1589**:168-180.

Ushijima H, Tanaka K, Takeda M, Katsu T, Mima S and Mizushima T (2005) Geranylgeranylacetone protects membranes against nonsteroidal anti-inflammatory drugs. *Mol Pharmacol* **68**:1156-1161.

Wirth D, Bureau F, Melotte D, Christians E and Gustin P (2004) Evidence for a role of heat shock factor 1 in inhibition of NF-kappaB pathway during heat shock response-mediated lung protection. *Am J Physiol Lung Cell Mol Physiol* **287**:L953-961.

Xiao X, Zuo X, Davis AA, McMillan DR, Curry BB, Richardson JA and Benjamin IJ (1999) HSF1 is required for extra-embryonic development, postnatal growth and protection during inflammatory responses in mice. *EMBO J* **18**:5943-5952.

Yan LJ, Christians ES, Liu L, Xiao X, Sohal RS and Benjamin IJ (2002) Mouse heat shock transcription factor 1 deficiency alters cardiac redox homeostasis and increases mitochondrial oxidative damage. *EMBO J* **21**:5164-5172.

Yasuda H, Shichinohe H, Kuroda S, Ishikawa T and Iwasaki Y (2005) Neuroprotective effect of a heat shock protein inducer, geranylgeranylacetone in permanent focal cerebral ischemia. *Brain Res* **1032**:176-182.

Zeniya A, Otaka M, Itoh H, Kuwabara T, Fujimori S, Otani S, Tashima Y and Masamune O (1995) Induction and intracellular localization of a 72-kDa heat shock protein in rat gastric mucosa after water-immersion stress. *J Gastroenterol* **30**:572-577.

Zheng H and Li Z (2004) Cutting edge: cross-presentation of cell-associated antigens to MHC class I molecule is regulated by a major transcription factor for heat shock proteins. *J Immunol* **173**:5929-5933.

FOOTNOTES

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FIGURE LEGENDS

Fig. 1.

Production of gastric lesions in wild-type and HSF1-null mice. Wild-type (A-C) and HSF1-null mice (B-C) were orally administered the indicated doses of ethanol (A-B) or hydrochloric acid (C). After 4 h, the stomach was removed and scored for hemorrhagic damage. Values are mean ± S.E.M. (n=4-6). $^{**}P<0.01$.

Fig. 2.

HSF1-dependent up-regulation of gastric mucosal HSPs induced by ethanol. Wild-type and HSF1-null mice were orally administered the indicated doses of ethanol (A, B). After 4 h, the gastric mucosa was removed and total RNA was extracted. Samples were subjected to real-time RT-PCR, using a specific primer for each gene. Values normalized to the *GAPDH* gene, and expressed relative to the control sample (i.e. wild-type mice not exposed to ethanol), are given as the mean ± S.E.M. (n=3). $^{***}P<0.001$, $^{**}P<0.01$, $^{*}P<0.05$ (A). After 4 h, the gastric mucosa was removed, and protein

extracts were prepared and analysed by immuno-blotting with an antibody against HSP25, HSP60, HSP70, HSP90, or actin. The band intensity of each HSP was determined by densitometric scanning, normalized with its respective actin intensity and the value of the ratio of band intensity between each HSP and the actin was shown under the band (B).

Fig. 3.

Ethanol-induced HSF1-dependent up-regulation of HSP70 in gastric mucosa. Wild-type (A) and HSF1-null mice (B) were orally administered the indicated doses of ethanol. After 4 h, sections of gastric tissues were prepared and subjected to histological examination (HE) and immunohistochemical analysis with an antibody against HSP70.

Fig. 4.

Induction of apoptosis by ethanol in gastric mucosa. Wild-type and HSF1-null mice were orally administered the indicated doses of ethanol. After 4 h, sections of gastric tissues were prepared and subjected to TUNEL assay.

Fig. 5.

Effect of increase or decrease in the expression of HSP70 on ethanol-induced cell death. AGS cells were transfected with plasmid with the *hsp70* gene (A, B) or siRNA for the *hsp70* gene (siHSP70) or non-silencing siRNA (ns) (C, D). After 24 h, cells were incubated with or without 7% ethanol for 1 h. The levels of HSP70 and actin were estimated by immuno-blotting with an antibody against HSP70 or actin (A, C). Cell viability was determined by MTT method. Values shown are mean ± S.D. (n=3). ***$P<0.001$; *$P<0.05$. n.s., not significant.

Fig. 6.

Gastric acid secretion, GMBF and PGE_2 levels in HSF1-null mice. The pylorus-ligated wild-type mice were administered 20 mg/kg histamine (*s.c.*) (A). Wild-type and HSF1-

null mice were orally administered 40% ethanol, and 2 h later the pylorus was ligated (B). A further two hours after the pylorus ligation, the acidity of the gastric contents was measured, as described in materials and methods (A, B) (n=3-4). After exposure of the stomach and GMBF stabilization, PGE_2 (0.03 mg/kg) was administered intravenously via the tail vein of wild-type and HSF1-null mice and changes in GMBF were monitored (C) (n=6). Wild-type and HSF1-null mice were orally administered 40% ethanol. After 4 h, the gastric mucosal PGE_2 level was determined by ELISA (D) (n=3-4). n.s., not significant.

Fig. 7.

Effect of ethanol and/or GGA on expression of HSP70 and production of gastric lesions. Wild-type (A, C) and HSF1-null (B, C) mice were orally pre-administered 200 mg/kg GGA (10 ml/kg as emulsion with 5% gum arabic), 1 h after which they were orally administered with the indicated doses of ethanol. After 4 h, sections of gastric tissues were prepared and subjected to histological examination (HE) and immunohistochemical analysis with an antibody against HSP70 (A, B). After 4 h, the

stomach was removed and scored for hemorrhagic damage. Values are mean ± S.E.M. (n=3-6). *P<0.05. n.s., not significant (C).